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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Appln of : Annie MEINIEL et al.

Serial No.09/462,909
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Olga N

Group Art Unit : 1646
Examiner : CHERNYSHEV,

For : Novel peptides and polypeptides useful for regenerating the nervous system

DECLARATION UNDER 37 CFR § 1.132

Honorable Commission of
Patent and Trademarks
Washington, DC 20231

Sir :

I, Dr S. GOBRON, declare as follows :

I am co-founder and chief scientific officer of Nucleica SA, a biotechnology company engaged in the development of new therapeutics and diagnostics. Previously, I obtained a PhD in Clermont-Ferrand (France) on the characterization of a new gene, *SCO-spondin*, and its encoding protein observed in the central nervous system of various animals. This work was the subject of different scientific publications as indicated below:

- Gobron S, Creveaux I, Meiniel R, Didier R, Herbet A, Bamdad M, El Bitar F, Dastugue B, Meiniel A. Subcommissural organ/Reissner's fiber complex: characterization of SCO-spondin, a glycoprotein with potent activity on neurite outgrowth. *Glia*. 2000 Nov;32(2):177-91.

- Gobron S, Creveaux I, Meiniel R, Didier R, Dastugue B, Meiniel A. SCO-spondin is evolutionarily conserved in the central nervous system of the chordate phylum. *Neuroscience*. 1999 Jan;88(2):655-64.

- Creveaux I, Gobron S, Meiniel R, Dastugue B, Meiniel A. Complex expression pattern of the SCO-spondin gene in the bovine subcommissural organ: toward an explanation for Reissner's fiber complexity? *Brain Res Mol Brain Res.* 1998 Mar 30;55(1):45-53.

- Gobron S, Monneric H, Meiniel R, Creveaux I, Lehmann W, Lamalle D, Dastugue B, Meiniel A. SCO-spondin: a new member of the thrombospondin family secreted by the subcommissural organ is a candidate in the modulation of neuronal aggregation. *J Cell Sci.* 1996 May;109 ( Pt 5):1053-61.

- Meiniel A, Meiniel R, Didier R, Creveaux I, Gobron S, Monneric H, Dastugue B. The subcommissural organ and Reissner's fiber complex. An enigma in the central nervous system? *Prog Histochem Cytochem.* 1996;30(2):1-66.

Since 2001, I am also vice-president of BIOMAC, a professional association of biotechnology companies of the Espace Central region in the heart of France allowing development and network of different actors of the sector, and representing a force of proposition to provide an economically favorable environment for biotechnology. Representation of the participating firms in international meeting, such as the annually world's largest biotechnology conference organized by Bio ("Biotechnology industry organization", USA), is also one of the primary aims of BIOMAC.

I am well aware of the contents of the present application as I am the inventor of it.
I read and understood the Official Action dated 10/18/2002.

Under my supervision, the following experiments were made to assess the properties of peptides of the invention as regenerative agents, particularly for the indication of spinal cord injury:

The aim of the study was to assess the neuroprotective and axonal regrowth properties of peptides of the invention *in vivo* after spinal cord injury (SCI) to address the potential use of this protein as a therapeutical indication in case of spinal trauma. With regards to the pathophysiology of spinal trauma, a cascade of events is involved. The primary physical injury disrupts the membrane of neurons, glial cells and microvessels, destroying axons and myelin in the longitudinal tracts. Injury-promoting factors are then released leading to secondary injury. Among these factors are included oedema formation,

neuronal death and glial cells proliferation, excitotoxicity, intracellular calcium accumulation along with free radicals release.

Unlike neuroprotective agent, such as antiglutamate, which may exert their efficacy through a single administration, agents acting as growth factors, on the other hand, need to be present over a longer period of time to exert their effect. To further confirm the efficacy of peptides of the invention in inducing neuritic outgrowth *in vivo*, pep1 (WSGWSSCSRSCG) was delivered within a collagen device at the site of lesion, previously made by a gentle aspiration of the dorsal funiculi and of the grey matter of the dorsal horns at thoracic level (T10-T11) of the spinal cord. The detection of structural markers such as neurofilament was assessed within the collagen device to look for the regrowth of nerve fibers from the proximal towards the distal stumps of the lesion.

Materials and Method

After opening the skin, the paravertebral muscles were retracted from the posterior arches of the vertebrae. Using an operating microscope, a laminectomy was performed from T11 to T12 thoracic vertebrae. A 5 mm longitudinal incision was made in the dura mater, allowing aspiration by gentle suction of the dorsal funiculi and of the dorsal horns of the grey matter. The resulting cavity was about 5 mm in length and 1 mm in depth. The cavity was packed with Gelfoam (Upjohn) to achieve hemostasis.

*Drug Administration*

A 5 mm length collagen channel (Human placental collagen type IV/Ivox. nerve guide channels, 1 mm inner diameter; Imedex™, SADUC, Chanopost, France) filled with 10 µl of saline (Control) or 10 µl of 33 mg/ml pep1 solution (sequence of pep1: WSGWSSCSRSCG) was inserted longitudinally into the cavity previously formed. Closure of the dura matter was done with three 10-0 sutures. The surgical wound was sutured and the skin closed with wound clips. The animals were placed in individual cages in a heated environment (28 °C to 30 °C) with food and water *ad libitum*.

*Immunohistochemistry*

Spinal cords perfused with Paraformaldehyde (PFA) were removed, post-fixed 24 hours in PFA, transferred to a 30% sucrose solution in PBS for 3 days, frozen in isopentane at- 50 °C and embedded in O.C.T. compound (Tissue Tek, Elkhart, IN). Cryostat sections

were cut and fixed onto glass slides precoated with 2% aminopropyltriethoxylisane (Fluka, Buchs, Switzerland).

Sections were incubated (overnight, at room temperature) with primary antibodies against neurofilament to identify axons (1:200 in 0.01 M PBS containing 1% triton X- 100 and 10% normal goat serum, Dako, Glostrup, Denmark). The next day, sections were washed and incubated for two hours with Cy3-conjugated goat anti mouse antibodies (diluted at 1:400 in 0.01 M PBS containing 1% triton X-100 and 10% normal goat serum, Interchim Montlucon, France). Slides were washed, mounted and examined using Nikon fluorescence microscopy.

*Morphometrical analysis*

Animals were sacrificed ten days after surgery and their spinal cord were processed for NF-immuno-histological analysis to explore axonal growth into the collagen channels.

Results

In all animals of the control group, the collagen tube filled with saline solution was devoided of connective tissue and neurofilament-immunoreactive (NF-IR) fibers ,as seen on figure 1 (Fig.1, document attached).

In contrast, in the pep1-treated group (Fig.2, document attached), newly formed tissue is observed inside the collagen tubes, this tissue coming from the rostral (r) as well as the caudal (c) spinal stumps. This connective tissue formation was intimately fused with the lesioned spinal cord. In two animals, NF-IR fibers were able to regenerate in the collagen channels for up to 2 mm (Fig 2 C, document attached).

The immunodetection of neurofilament revealed the regrowth of denervated fibers in the rat spinal cord. Fibers along with connective tissue were able to invade the channel filled with pep1 from both stumps of the cord. This response was specific to the pep1-treated group with more or less effect depending on animals.

These data demonstrate that pep1 was able to induce the regrowth of axons *in vivo* along with the development of connective tissue in the lesioned spinal cord . The peptides of the invention are therefore regenerative agents for the indication of spinal injury.

I submit that peptides of the present invention have been identified as candidate regenerative agents in the central nervous system. This is a part of our works on the characterization of a specific product observed in the central nervous system of vertebrates. These works allowed isolating and characterizing the SCO-spondin protein, with specific tools such as specific antibodies, as a part of the Reissner's fiber. Furthermore, the patent application is based on our work on the activity of the protein, in nervous system cells cultures which has shown neuroprotection, modulation of cells interactions and neuritic outgrowth, and which provides a practical utility of the protein in case of neuroregeneration. Thus, I submit that there is a clear and "definitive" biological activity of the peptides of the invention, on the basis of molecular features, *in vitro* tests and *in vivo* studies which all argue for a practical utility of the peptides of the invention in the neuroregeneration.

As indicated in Monnerie et al., 1998, various peptides deduced from SCO-spondin has been tested for their biological activity on nervous cells, and interestingly, only peptides of the present invention (peptides derived from thrombospondin type I motifs of SCO-spondin) have been shown as specifically actives on cortical and/or spinal neurons. Among biological activities of SCO-spondin peptides of the present invention are enhancements of neuronal survival and neuritic outgrowth, two phenomenons being among key targets for therapies of various neurodegenerative diseases and particularly in case of spinal cord injury. As mentionned by Hoffer and Olson, 1997, "adult CNS axons are able to elongate efficiently if given the appropriate environment", and peptides of the present invention can be an appropriate environment since in the presence of pep1 regenerative tissue is observed as previously shown. Thus, in treated animals, e.g. animals with collagen tube filling with pep1(WSGWSSCSRSCG, choosen as the most active sequence tested *in vitro*), a neuritic outgrowth has been clearly observed in the tube indicating the capacities of pep1to act *in vivo* as a regenerative agent. Thus, peptides of the present invention are able to induce the regeneration of nervous cells *in vivo*. Therefore, administration of peptides of the present invention will be useful in case of therapy of nervous system diseases and particularly in case of nervous injury such as spinal cord injury.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these

statements and the like so made are punishable by fine and imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted

Date : 01/07/2003

By : Stéphane GOBRON, PhD